Mechanism of secB-dependent preprotein targeting to the translocase

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Chapter 6

Summary and concluding remarks

A revised version of this chapter was published in *Current Opinion in Microbiology* **1**, 216-222 (1998)

Introduction

Protein translocation across the cytoplasmic membrane of *Escherichia coli* is mediated by a cytosolic chaperone, SecB, and a multisubunit integral membrane protein complex, termed translocase. This large complex consists of a peripheral ATPase, SecA, and at least five integral membrane proteins (*i.e.* SecY, SecE, SecG, SecD and SecF). The precursor proteins are equiped with a N-terminal extension, *i.e.* the signal sequence, that is recognized by targeting factors to direct them to the translocation site. Translocation depends on the the energy of ATP hydrolysis by SecA and is stimulated by the protonmotive force (Δp) across the membrane.

Preprotein targeting to the membrane

SecB interaction with preproteins

SecB is a molecular chaperone with a dual function in preprotein translocation. It keeps preproteins in a loosely folded conformation that is competent for translocation and targets them to the membrane-bound SecA subunit of the translocase. *In vitro*, SecB binds without specificity, provided that the substrate is in a nonnative state. *In vivo*, however, SecB is very selective and binds mainly to a subset of nascent preproteins. This high selectivity without specificity was explained by the kinetic partitioning model which predicts that SecB distinguishes between cytosolic and precursor proteins by the differences in folding rate (Hardy and Randall, 1991; Randall and Hardy, 1995). This hypothesis was based on the observation that *in vitro*, proteins with a signal sequence have a lower folding rate compared to those without a signal sequence (Park *et al.*, 1989). Cytosolic proteins would fold fast and preproteins would fold slow giving SecB time to stably interact. However, as described in Chapter 2, the interaction between SecB and preproteins is readily reversible, diffusion-limited and occurs at rates well beyond that of typical folding. This implies that it is unlikely that SecB distinguishes between cytosolic and precursor proteins on the basis of the difference in folding rates. Based on the association rates, both type of proteins fold too slow to escape interaction with SecB and are, according to the kinetic partitioning theory, potential substrates for SecB. In principal, SecB would be able to interact with any newly synthesized protein. This is not the case since only precursor proteins are found to interact with SecB (Kumamoto, 1989). Another observation that argues against the kinetic partitioning model is that the translocation of precursor proteins which lack a signal sequence is totally SecB-dependent (Derman *et al.*, 1993; Flower *et al.*, 1994; Prinz *et al.*, 1996; Chapter 4). These proteins miss the folding rate suppressing effect of the signal sequence but still interact with SecB. Moreover, the folding rate may not be an issue at all for *in vivo* SecB binding, as SecB binds to nascent chains (Kumamoto and Francetić, 1993: Randall *et al.*, 1997) that are generally believed to lack any stable folded domains. Therefore, *in vivo*, the binding affinity for SecB is more likely to be decisive for a stable interaction rather than the rate of folding.

What determines the binding affinity for SecB is a matter of speculation. The preprotein-binding site on SecB is localized in a small region on SecB and consists of mainly hydrophobic residues, which alternate with the residues involved in SecA binding (see below). SecB has a high content of β-structure (Fasman *et al.*, 1995) and exists as a tetramer (Smith *et al.*, 1996), which makes it possible that the residues involved in preprotein binding together form a hydrophobic surface. This coincides with the notice that SecB binds nonnative proteins, which are generally believed to expose hydrophobic patches towards the outside. Also, in Chapter 2, it is shown that the interaction between SecB and the preprotein involves hydrophobic interaction. The site on a preprotein where SecB binds seems to be very

specific since the proteolytic fragments obtained after digestion of the preprotein bound to SecB are discrete and uniform (*e.g.* Khisty *et al.*, 1995). This means that SecB probably recognizes a domain in the preprotein with specific, possibly secondary, structural features. In this respect, it is interesting to note that preproteins indeed contain secondary and even tertiary structure when bound to SecB (Lecker *et al.*, 1990; Breukink *et al.*, 1992). Taking in account the hydrophobic nature of the preprotein-binding site on SecB, this domain is most likely also hydrophobic and the residues involved in SecB binding may, in analogy to the preprotein-binding site on SecB, alternate with other residues.

Overlapping preprotein- and SecA-binding sites on SecB

After forming a stable interaction between SecB and the preprotein, this binary complex has to dock on the SecA subunit at the translocation site. Membrane-bound SecA and SecB have a high affinity for each other. This implies that on both proteins a mutual binding site is present. The identification of the SecA-binding site on SecB is described in Chapter 4. Earlier work uncovered several mutations in SecB that influenced the protein translocation efficiency *in vivo* (Gannon and Kumamoto, 1993; Kimsey *et al.*, 1995). These mutations could be classified into three classes. Mutations from class 1 result in SecB proteins that are impaired in preprotein binding, but cause only a mild defect in the rate of maltose-binding protein (MBP) export as judges from pulse-chase experiments. Class 2 mutations are characterized by a severe slow-down in the rate of MBP export without any effect on the preMBP/SecB complex formation. The third class (class 3) consists of SecB mutations that render the SecB protein unable to support growth of *E. coli* on rich media, indicating that there is no SecB activity at all. The mutations cluster in two regions of SecB with region 1 composed of residues 74-80 and region 2 of residues 20-24. Class 1 and 3 mutations are restricted to region 1, whereas class 2 mutations are found in both regions. It has been found that class 1 mutations are impaired in the preprotein binding, indicating that these residues are involved in the SecB/preprotein complex formation (Kimsey *et al.*, 1995). Class 3 mutations result in a SecB protein that is rapidly degraded since hardly any SecB could be immunodetected in these strains. This class only consists of proline substitutions which probably cause a severe disturbance in the overall structure and stability of SecB. In Chapter 4, it is shown that the SecB proteins with the substitutions Leu75→Gln and Glu77→Lys, both belonging to class 2, still recognize preprotein, but are disturbed in the recognition of SecA. This indicates that these two residues, and most likely all other residues belonging to class 2, are part of the SecA-binding site on SecB. The residues involved in preprotein binding and those for SecA binding in region 1 alternate. When a β-structural conformation is assumed, the residues involved in SecA binding are located on one side of the strand, while those involved in preprotein binding face the other side.

The SecB-binding site on SecA

In Chapter 3, the identification of the SecB-binding site on SecA is described. This site is found to reside in the extreme carboxy-terminus of SecA and can functionally be transferred onto the C-terminus of an unrelated protein. Deletion of this domain renders a SecA protein (SecAN880) that is still active in protein translocation, but that is unable to bind SecB. Heterodimers of wild-type SecA and SecAN880 are defective in SecB binding, demonstrating that both carboxy-termini of the SecA dimer are needed to form a genuine SecB-binding site. The SecB-binding domain is composed of only 20 amino acid residues with a high content of lysyl and arginyl residues giving rise to a strong electropositive surface with a predicted pI value near 10. The positive charge may facilitate the interaction with SecB since the SecA-binding site on SecB is, at least partially, composed of negatively charged residues (see above). This strongly suggests that the SecA-SecB binding is mediated via electrostatic interaction. Other features of the SecB-binding site on SecA are three cysteinyl residues and several glycyl and prolyl residues. The function of the cysteinyl residues is unknown, but the presence of the glycyl and prolyl residues indicates that this domain is highly flexible. Recently, it has been found that the binding of SecB to this domain is dependent on the presence of zinc ions (P. Fekkes, J.G. de Wit and A.J.M. Driessen, manuscript in preparation). The fusion between the SecB-binding domain and GST is unable to bind SecB in the

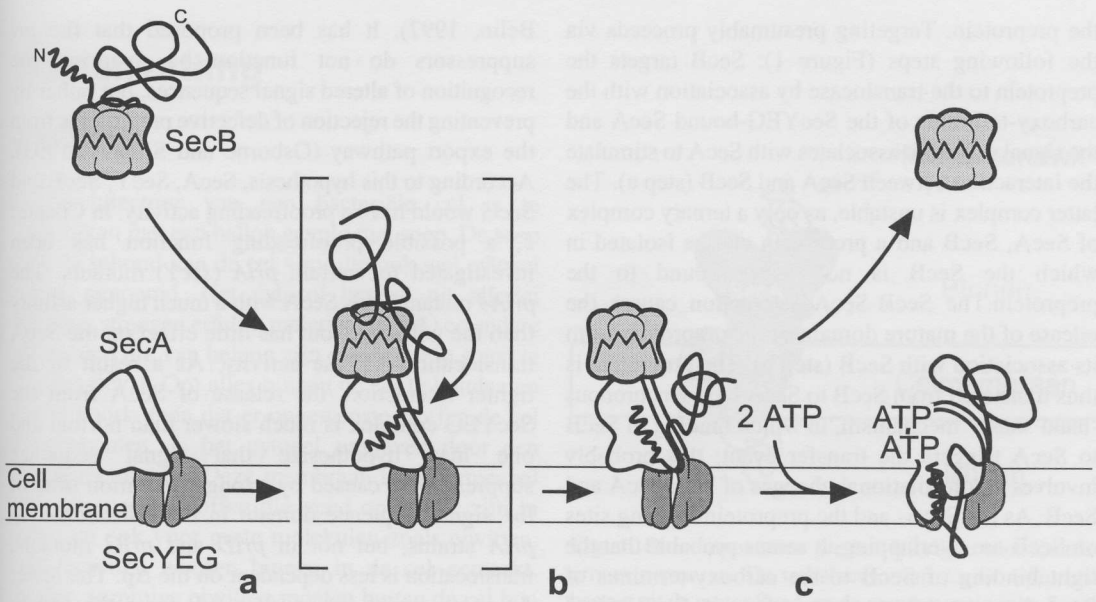

Fig. 1. Schematic representation of the initial stages of preprotein translocation. (**a**) SecB targets the preprotein to the SecYEG-bound SecA and the signal sequence associates with SecA to stimulate the interaction between SecA and SecB. (**b**) The SecB-SecA interaction causes the release of the mature domain of the preprotein from SecB. The zig-zag symbol represents the region in SecB that associates with both SecA and preprotein, and changes conformation upon SecA interaction. (**c**) After SecA binds ATP, which results in conformational changes in SecA, SecB is released from SecA and translocation of the preprotein is initiated.

presence of, or after treatment with EDTA or the more specific zinc chelator tetrakis-(2-pyridylmethyl)ethylenediamine (TPEN). Readdition of zinc-, copper- or, to a lesser extence, nickel ions restored the SecB-binding capacity. Atomic adsorption revealed that about one zinc atom per GST-fusion protein is present. The SecB-binding domain of *E. coli* SecA harbors three conserved cysteines and one histidine, which together may be involved in the coordination of the zinc atom. The SecB-binding domain is highly conserved among the bacterial SecA proteins, except for *Streptomyces*, *Mycobacterium*, and *Mycoplasma* species. This hints to the presence of proteins in other bacteria with SecB-like properties that need a docking place at the membrane. Until now, only in Gram-negative bacteria SecB-homologues have been identified.

Preprotein transfer from SecB to SecA

The importance of the targeting function of SecB is evident when the preprotein signal sequence is either mutated (Francetić and Kumamoto, 1996) or completely removed (Flower *et al.*, 1994), thereby reducing the targeting information contained in the preprotein. Such proteins are dependent on SecB for translocation. In Chapter 4, it is shown that when the SecB-binding domain of SecA is removed, the translocation of preproteins with a defective signal sequence is blocked by SecB instead of stimulated. This is indicative for a need for SecB binding by SecA for preprotein transfer. The interaction between SecB and SecA in the cytosol is of a low affinity (Den Blaauwen *et al.*, 1997), whereas SecB binds with high affinity to the SecYEG-bound SecA (Hartl *et al.*, 1990). In the presence of a preprotein with a functional signal sequence, the SecB-SecA binding affinity is even enhanced. This phenomenon is caused by the binding of the preprotein signal sequence to SecA. SecB-mediated targeting of the preprotein to SecA thus also occurs via the signal sequence which is available for SecA interaction as SecB has been shown to bind only the mature part of

the preprotein. Targeting presumably proceeds via the following steps (Figure 1): SecB targets the preprotein to the translocase by association with the carboxy-terminus of the SecYEG-bound SecA and the signal sequence associates with SecA to stimulate the interaction between SecA and SecB (step a). The latter complex is unstable, as only a ternary complex of SecA, SecB and a preprotein can be isolated in which the SecB is no longer bound to the preprotein.The SecB-SecA interaction causes the release of the mature domain of the preprotein from its association with SecB (step b). The preprotein is thus transfered from SecB to SecA by a synchronous 'hand-shake' mechanism, in which binding of SecB to SecA triggers the transfer event; this probably involves conformational changes of both SecA and SecB. As the SecA- and the preprotein-binding sites on SecB are overlapping, it seems probable that the tight binding of SecB to the carboxy-terminus of SecA dissociates the preprotein from its SecB-bound state. An important implication of this mechanism is that SecB bound at the translocase will be unable to accept new cargo as long as it remains bound to SecA. Only after the initiation of translocation by the binding of ATP to SecA SecB is released from the membrane to bind a new preprotein in the cytosol (step c).

Signal sequence proofreading at the initiation of translocation

From the SecA-SecYEG-bound state, preprotein translocation is initiated by the binding of ATP to SecA, allowing the membrane-insertion of the signal sequence and part of the mature preprotein region likely as a hairpin-like structure (Schiebel *et al.*, 1991). The so-called *prl* (for *pr*otein *l*ocalization) class of mutants, which are all isolated as suppressors of signal sequence mutations, have been found in SecA (*prlD*), SecY (*prlA*), SecE (*prlG*) and more recently, in SecG (*prlH*) (see references in Huie and Silhavy, 1995; Prinz *et al.*, 1996; Bost and Belin, 1997). It has been proposed that the *prl* suppressors do not function by restoring the recognition of altered signal sequences, but rather by preventing the rejection of defective preproteins from the export pathway (Osborne and Silhavy, 1993). According to this hypothesis, SecA, SecY, SecE and SecG would have a proofreading activity. In Chapter 5, a possible proofreading function has been investigated for certain *prlA* (*secY*) mutants. The *prlA4* mutant binds SecA with a much higher affinity than the wild-type, but has little effect on the SecA translocation-ATPase activity. As a result of the tighter interaction, the release of SecA from the SecYEG complex is much slower than normal and one may hypothesize that signal sequence suppression is caused by a longer retention time of the signal sequence domain in the translocase. In *prlA* strains, but not in *prlD* and *prlG* mutants, translocation is less dependent on the Δp. The lower Δp requirement may well result from the tighter SecA interaction just like high levels of SecA or ATP can suppress the Δp requirement for translocation.

Concluding remarks

Besides keeping the precursor protein in a translocation-competent state, the targeting function of SecB is an important feature. These functions can be separated by a single mutation in this chaperone. SecB itself cannot utilize ATP. It employs for that purpose SecA and agrees with a role as cochaperone dedicated to the targeting of a subset of preproteins to the cytoplasmic membrane of *E. coli*. The studies presented in this thesis allowed us to reconstruct in great detail the events that occur from the moment of preprotein binding to SecB to the inititiation of translocation upon the binding of ATP.